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Original Paper

Optimization of callus induction protocol from leaf explants of *Portulaca oleracea* and assessment of fatty acid profiles

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Abstract

A protocol for optimization of callus culture establishment from leaf explants of *Portulaca oleracea* L. was developed. The effect of ethyl methanesulphonate (EMS) on callus induction and synthesis of fatty acids was evaluated. Callus culture was initiated from leaf explants on Murashige and Skoog's medium supplemented with 2,4-dichlorophenoxy acetic acid (2,4-D), a combination of 2,4-D + 6-benzyl adenine (BA) or 2,4-D + kinetin. The maximum callus biomass was obtained at 2.5 µM BA + 2.5 µM 2,4-D (20.22 g leaf explant^{-1} fresh weight). The leaves treated with EMS (0.1 to 0.4% h^{-1}) differentiated callus on this optimized medium combination. The gas chromatography-mass spectrometry analysis of fatty acids indicated that the leaves had a high linolenic acid content (17.72%). The callus cultures synthesized heptadecanoic, oleic, and tricosanoic acids, which were otherwise absent in the leaves. Eicosanoic and docosanoic acids in callus cultures were 2.78 and 3.18 times higher than their content in the leaves, respectively. The diversity of fatty acids in treated callus cultures decreased with increased EMS concentration, but the content of a few individual fatty acids was enhanced. Callus at the dose of 0.1% EMS synthesized linoleic acid, which was two times higher than in the untreated callus, while at 0.2% EMS stearic acid was synthesized, which was absent in the untreated callus. It was concluded that the callus of *P. oleracea* accumulated different fatty acids, and EMS treatment enhanced the content of a few fatty acids in the cultures.

Key words: auxin, callus, cytokinin, ethyl methane sulphonate, omega fatty acids, purslane, plant tissue culture.
Abbreviations: 2,4-D, 2,4-dichlorophenoxy acetic acid; BA, 6-benzyladenine; BF_3, boron trifluoride; CGI, callus growth index; DW, dry weight; EMS, ethyl methanesulphonate; FAME, fatty acid methyl ester; FW, fresh weight; KIN, kinetin; LD_{50}, lethal dose 50; MS, Murashige and Skoog's; OFAs, omega fatty acids; PGR, plant growth regulator; PUFAs, polyunsaturated fatty acids.

Introduction

Portulaca oleracea L., commonly known as purslane, belongs to the Portulacaceae family. It is a common succulent found in sunny areas alongside fields, lawns and wastelands, predominantly in temperate regions, such as India and Mediterranean countries (Anonymous 2003). Purslane is highly nutritious and possesses several medicinal properties, including anti-diabetic (Lee et al. 2020); anti-inflammatory (Chang et al. 2020); anticancer; antioxidant and antimicrobial effects (Zhou et al. 2015). Being a rich source of K, Mg, Ca, vitamin A and C, beta carotenes, alkaloids, flavonoids, terpenoids, and sterols, the plant has been extensively used in the pharmaceutical industry (Uddin et al. 2014; Zhou et al. 2015; Petropolous et al. 2019; Liu et al. 2024). This plant is also rich in essential dietary fatty acids such as linoleic and linolenic acids (Srivastava and Joshi 2021), which are required for normal growth (Glick, Fisher 2013).

The essential fatty acids, such as linoleic and linolenic acids are not synthesised adequately within the human body and need to be ingested through regular diet or supplements (Glick, Fischer 2013; Parasannanavar et al. 2021). These essential fatty acids are classified into two families i.e. omega-3 and omega-6 fatty acids. Omega-3 fatty acids have a double bond at the third position of carbon atom from the methyl end of the fatty acid molecule, whereas omega-6 fatty acids have a double bond at the sixth carbon atom starting from the same end of the fatty acid molecule (Kaur et al 2014). These omega fatty acids (OFAs) are crucial for proper brain development, better vision, cognitive behaviour and reduce the risk of cardio vascular diseases (Parasannanavar et al. 2021). A report by the Indian Council of Medical Research (2010) in India, recommends a total fat intake of 25 to 50 g day^{-1} in children and adolescents aged 4 to17 years. Studies have indicated low intake of OFAs and polyunsaturated fatty acids (PUFAs) in school children (Thompson et al. 2019; Parasannanavar

et al. 2021). In India, the low intake of OFAs is due to a vegetarian diet dominated by carbohydrates such as wheat and rice. Thus, it is essential to explore plant-based sources of OFAs. *P. oleracea* has been reported to contain high levels of OFAs, such as linoleic and linolenic acids (Uddin et al. 2014; Mousavi, Niazmand 2017; Farag et al. 2019; Petropolous et al. 2019). However, the OFAs content in plants is generally lower than that in animals, such as fish. Enhancing the dietary fatty acid content in plants through research and development is necessary.

The metabolite content in plants can be modified using plant tissue culture techniques combined with biotechnological methods, such as elicitation and mutagenesis. *In vitro* mutagenesis, which integrates mutation breeding with *in vitro* propagation, offers several advantages, including a high rate of mutation frequency, uniform mutagen treatment of the explant, generation of disease-free plants, and requirement of less space to handle a large population of mutated cells (Penna et al. 2012). Chemical mutagens such as ethyl methanesulphonate (EMS) are effective in inducing desirable traits by causing specific genetic mutations (Watson et al. 2006). Previous studies have shown that EMS-induced mutations lead to several desirable characters in plants (Dalvi et al. 2021). New chrysanthemum and insect resistant eggplant mutants have been developed by in vitro mutagenesis (Islam et al. 2022; Nasri et al. 2022). Indirect organogenesis was obtained in *P. grandiflora* by treating the callus with mutagens EMS and NaN_3 (Bennani, Rossito Hassani 1997). In *P. oleracea* EMS treatment up-regulates fatty acid synthesis in *in vitro* shoots (Srivastava, Joshi 2021). However, the effect of mutagens on callus induction and subsequent fatty acid expression in *P. oleracea* has not yet been explored.

This study aimed to develop a protocol for the optimization of callus culture growth and to assess fatty acid accumulation in untreated and EMS-treated calluses. The study may be useful for developing suspension cultures for fatty acid extraction. This research provides insights into enhancing dietary fatty acid synthesis in *P. oleracea* calluses, contributing to the development of plant-based OFAs sources. To the best of our knowledge, this is the first study to investigate EMS-induced variation in fatty acid synthesis in callus cultures of *P. oleracea*.

Materials and methods

Sterilization and preparation of the explant for inoculation

Leaves were harvested from plants growing in the botanical garden of The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat. These leaves were washed under running tap water for one hour and then rinsed with a mild detergent to remove soil particles from the surface. The washed leaves were treated with 0.1% $HgCl_2$ for 3 min and rinsed with sterile deionized water (two to three times) for further surface sterilization.

Preparation of the medium

Murashige and Skoog's (1962) medium (MS medium) was used for the present study. This medium was supplemented with 3% sucrose and the following concentrations of plant growth regulators (PGRs): (i) individual 2,4-dichlorophenoxyacetic acid (2,4-D; 0.5, 2.5, 5, 10 µM); (ii) combined addition of 6-benzyladenine (BA; 0.5, 2.5, 5, 10 µM) and 2,4-D (0.5, 2.5, 5, 10 µM); (iii) combined addition of kinetin (KIN; 0.5, 2.5, 5 and 10 µM) and 2,4-D (0.5, 2.5, 5, 10 µM). The pH of the medium was adjusted to 5.8 by the addition of 0.1 N HCl or 0.5 N NaOH. The medium was solidified with 0.8% gelling agar and autoclaved at 205 kPa at 120 °C for 20 min.

Establishment of callus cultures

Surface-sterilized leaves were cut into 1 cm^2 explants and inoculated on different medium composition (supplemented with individual 2,4-D, BA plus 2,4-D, or KIN plus 2,4-D) for callus induction under a laminar hood. After inoculation, the cultures were maintained in culture rooms equipped with cool fluorescent white light at 25 ± 2 °C.

Calli from each combination were harvested after 8 weeks, and the fresh weight (FW) and dry weight (DW) was recorded. The fresh callus was washed with deionized water, dabbed with a paper towel, and kept in an oven at 40 °C for 48 h to obtain the DW of the callus. The medium that generated the highest callus biomass (FW/DW) was designated as the optimised medium for callus growth. This optimised medium was used to determine the callus growth index (CGI), which indicates the time at which the maximum biomass is obtained from the cultures. The CGI experiment was initiated with 1 g of callus, inoculated on the optimised concentration of MS medium, and six flasks were harvested every week. The CGI (for the FW and DW) was determined for 10 weeks and a graph was plotted for CGI versus time (weeks):

CGI = Final weight of the callus – Initial weight of callus / Initial weight of callus.

Treatment of EMS to the explant

The leaves were soaked in freshly prepared EMS in sterile distilled water at concentrations of 0.1, 0.2, 0.3, and 0.4% (*v*/*v*). The treatment flasks were kept on a shaker for 1 h at 60 rounds min^{-1} for uniform treatment of the leaves with EMS. The treated explants were rinsed with sterile distilled water (two to three times) and inoculated on an optimized callus-inducing medium. The FW and DW of the EMS-treated calli was recorded after 12 weeks.

Fatty acid extraction

The leaves and untreated calli (harvested from cultures at 8 weeks) were dried in an oven (40 °C for 48 h) and crushed to a fine powder with a pestle and mortar. Ten grams of dried and powdered leaves/untreated calli were used for

fatty acid extraction. The powdered samples were subjected to Soxhlet extraction in 500 mL of petroleum ether for 10 hours. The oil was esterified with 10% methanolic BF_3 (a 10% solution of BF_3 prepared in methanol) and filter sterilized through a 45 µm nylon filter membrane (Weston et al. 2008). A sample (1 µL) of fatty acid methyl ester (FAME) was injected in the gas chromatograph. Details of the gas chromatography and mass spectrometry program are provided in Srivastava and Joshi (2021). Extraction of fatty acids from EMS-treated calli (harvested after 12 weeks) was initiated from 2 g of callus. The volume of the reagents used for extraction was adjusted according to the sample weight (2 g). The fatty acid peaks in the gas chromatograms of the leaves, untreated callus and EMS-treated callus were compared with the peaks obtained from fatty acids of the standard (Supelco 37 component FAME mixture) and the mass spectra of the National Institute of Science and Technology library.

Statistical analysis

The callus induction experiments were conducted twice with 12 replicates each. The data for the two sets of experiments were pooled, and FW and DW were expressed as mean ± SE. Statistically significant difference within the means were analyzed by one-way ANOVA followed by a post hoc Tukey's HSD test, where $p \leq 0.05$. The gas chromatography of the samples was performed in triplicate, and the fatty acid content (obtained from DW of the samples) was expressed as the average of relative mean area (%) ± SE of the fatty acid peak. The difference in means of fatty acids within a sample was analyzed by an ANOVA test followed by a post hoc Tukey's HSD test.

Results

Induction of callus from explant

In the present study, calli were regenerated using 2,4-D individually and in combination with BA or KIN. A single concentration of 2,4-D at 0.5 µM formed two to three roots, whereas higher concentrations (2.5 to 10 µM) induced negligible callus formation. The combined concentration of BA (0.5 to 10 µM) with 0.5 µM 2,4-D induced variable responses. The concentration of 0.5 µM BA + 0.5 µM 2,4-D induced two to three roots; 2.5 to 5 µM BA + 0.5 µM 2,4-D differentiated shoot buds and callus, while 10 µM BA + 0.5 µM 2,4-D formed well-developed shoots (n = 2.34) and green callus after 8 weeks (Fig. 1A). The concentration of 2.5 to 10 µM BA with 2,4-D (2.5 to 10 µM) induced white compact callus, which appeared within 4 to 5 days of inoculation of the explant. Vigorous callus growth was observed as the explants were profusely covered with callus by 3rd week. High callus biomass was noted with BA (2.5 to 10 µM) and 2.5 µM 2,4-D. Callus biomass analysis revealed that optimum callus induction in terms of FW/DW was achieved at 2.5 µM BA and 2.5 µM 2,4D (20.2 g explant^{-1} in FW; Fig. 1B). Callus biomass decreased significantly at 10 µM BA and 10 µM 2,4-D. Thus, a high concentration of 2,4-D induced white compact callus with BA, but a low concentration of 2,4-D (0.5 µM) with BA induced root or shoot formation (Fig. 2). The explants responded profusely to callus induction, as all concentrations of BA + 2,4-D resulted in a 100% response rate with varying degrees of callus biomass after 8 weeks.

The response of the explants to the combined concentration of KIN and 2,4-D was similar to BA + 2,4-D

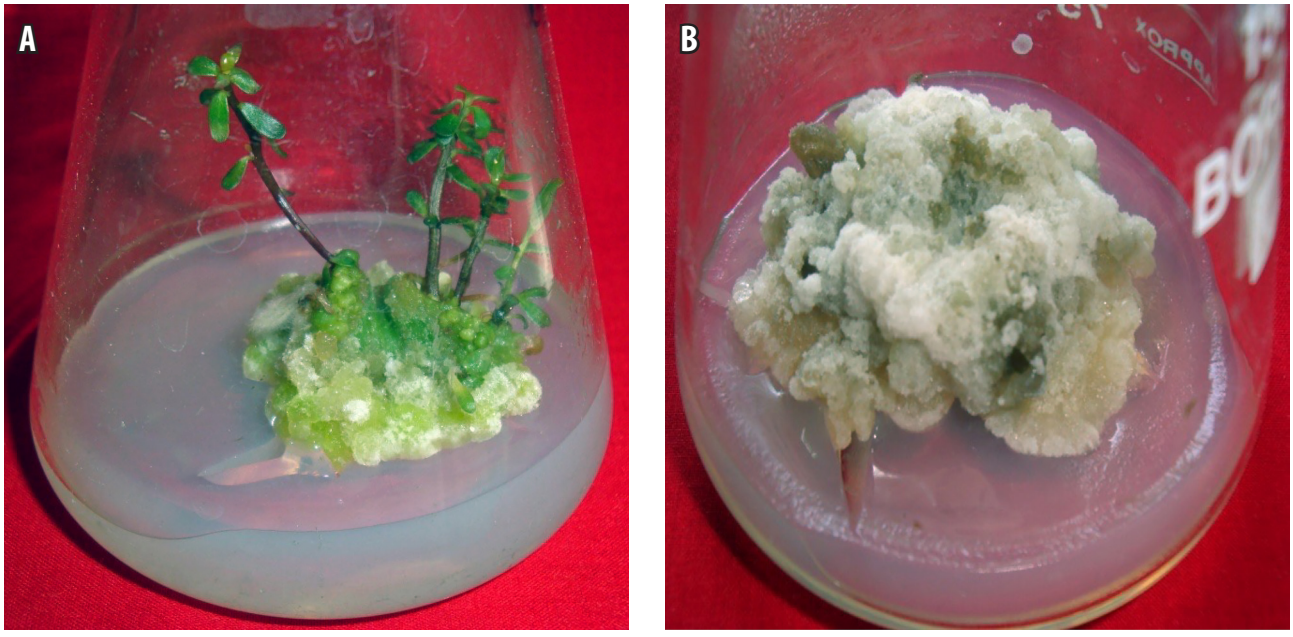

Fig. 1. Response of leaf explants of *P. oleracea* inoculated on MS medium supplemented with different PGRs after 8 weeks. A, regeneration of shoots at 10 µM BA+ 0.5 µM 2,4-D; B, induction of callus at 2.5 µM BA + 2.5 µM 2,4-D.

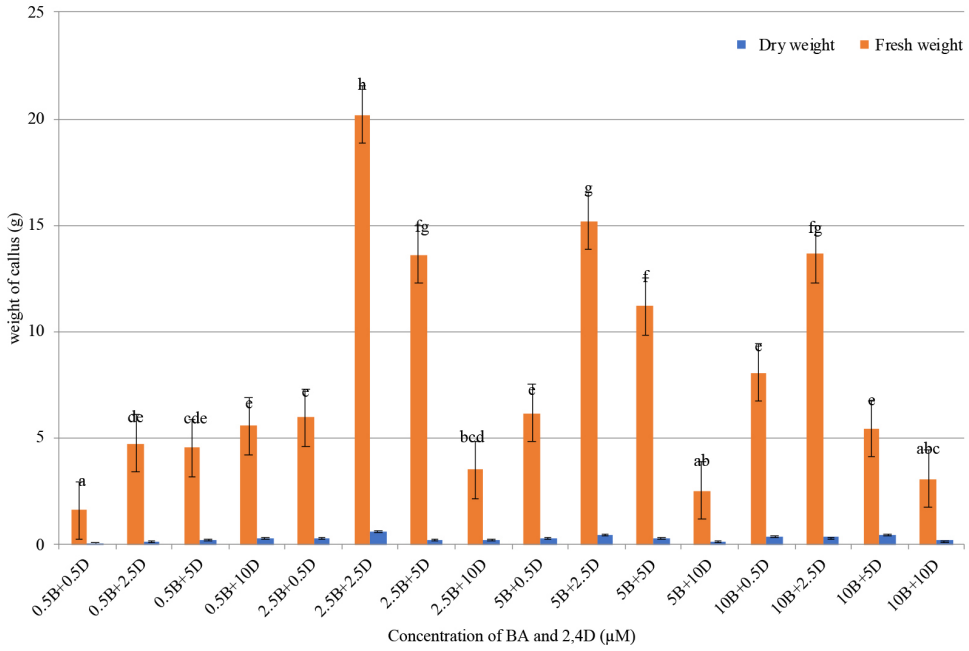

Fig. 2. Induction and growth of callus from leaf explants of *P. oleracea* on MS medium supplemented with different concentrations of BA and 2,4-D after 8 weeks. Means followed by the same letter are not statistically significantly different according to Tukey's HSD test ($n = 12$; $p \leq 0.05$).

after 8 weeks. The explants failed to induce any response when inoculated on the medium supplemented with 0.5 µM KIN + 2,4-D (0.5 to 10 µM). A concentration of KIN (2.5 to 5 µM) + 0.5 µM 2,4-D in the medium induced roots, shoots, and callus while 10 µM KIN + 0.5 µM 2,4-D induced yellow friable callus from the explant. The *in vitro* shoots formed in this medium composition (KIN and 2,4-D) had large leaves and small internodes. The medium supplemented with different concentration of KIN (0.5 to 10 µM) with 2.5 to 10 µM 2,4-D induced yellow or brown friable callus from the explant. The maximum FW callus induction in the combined concentration of KIN and 2,4-D was achieved with 5 µM KIN + 5 µM 2,4-D (9.6 g explant^{-1} in FW). A further increase in the concentration of both KIN and 2,4-D decreased the biomass (Fig. 3). The callus biomass obtained with KIN + 2,4-D was less than BA + 2,4-D combinations both on FW and DW parameters. These results suggest that BA/KIN + 2,4-D combinations were suitable for callus generation with the optimal concentration for callus induction in this plant being 2.5 µM BA + 2.5 µM 2,4-D.

CGI in terms of FW/DW was determined for the optimised concentration of callus induction (2.5 µM BA + 2.5 µM 2,4-D). Callus growth was slow during the first week, but increased steadily from the 2nd to the 7th week. A decline in growth was noted after the 7th week and by the 10th week; the callus turned brown (Fig. 4). Calluses were harvested in the 7th week of incubation for fatty acid extraction.

Effect of EMS on callus induction

The leaves were treated with EMS (0.1 to 0.4%) and inoculated on the optimized medium for callus induction (2.5 µM BA + 2.5 µM 2,4-D). Leaves swelled and induced calli from the proximal end of the explant. The entire explant was covered with white calli within 5 weeks. The texture of the callus was mostly friable; however, it was compact at a few sectors. Callus biomass (FW/DE was the highest at 0.2% (18.61 g explant^{-1} in FW). A further increase in EMS concentration decreased the biomass weight (Fig. 5).

Analysis of fatty acids in leaves, untreated and EMS-treated callus cultures

In the present study the content of different fatty acids was evaluated in the leaves, callus cultures (grown on MS medium supplemented with 2.5 µM BA and 2.5 µM 2,4-D) and callus cultures obtained from leaves treated with different concentrations of EMS. A variety of fatty acids were obtained from each sample. Palmitic acid was present in all the samples (leaves, untreated callus and EMS-treated callus) but its highest content was detected in untreated calli (14.94%). Heptadecanoic and oleic acid were present in the untreated callus while in the rest of the samples (leaves and EMS-treated calluses) it was absent. The content of OFAs such as linolenic acid was highest in leaves (17.72%), but declined considerably in untreated calli and they were absent in EMS-treated calli. A lower increase in the linoleic acid content was observed in the untreated calli than in the leaves. Synthesis of OFAs was severely affected in the EMS-treated calli as linoleic acid was synthesized at

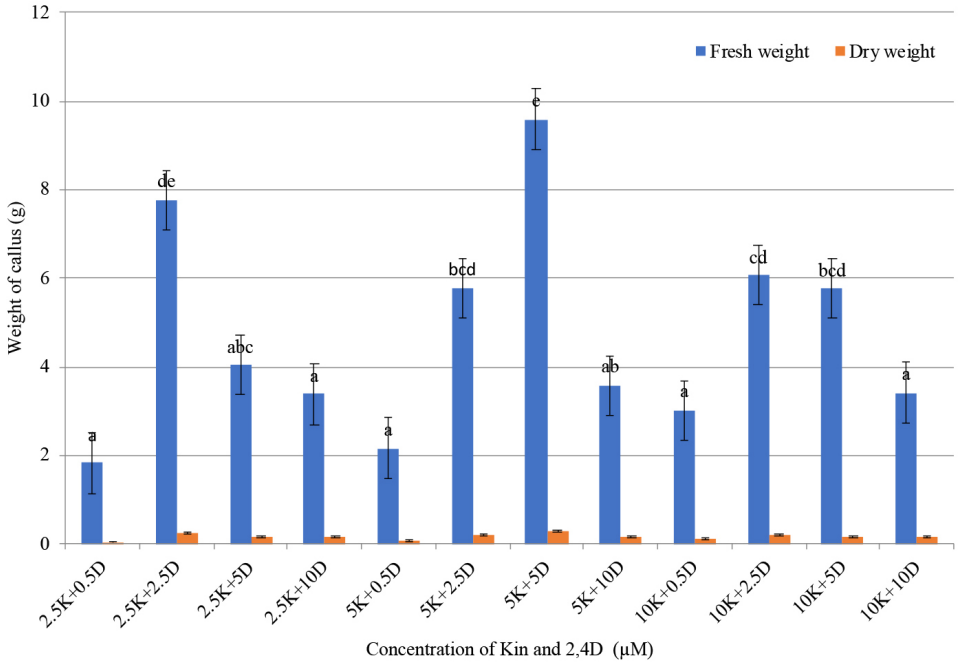

Fig. 3. Induction and growth of callus from leaf explants of *P. oleracea* on MS medium supplemented with different concentrations of KIN and 2,4-D after 8 weeks. Means followed by the same letter are not statistically significantly different according to Tukey's HSD test ($n = 12$; $p \leq 0.05$).

the 0.1% EMS dose (12.36%), while in the rest of the doses (0.2 and 0.3%) it was absent. None of the EMS-treated callus cultures synthesized linolenic acid. The content of PUFAs such as eicosanoic and docosanoic acid, was higher in the untreated callus than in the leaves. The docosanoic acid content of untreated callus was 3.18 times higher, than that of leaves, but its synthesis decreased drastically in the EMS-treated cultures.

Notably, tricosanoic acid was absent in the leaves, but the untreated calli synthesized this long-chain fatty acid. The callus grown at a dose of 0.1% EMS synthesised 1.6 times more tricosanoic acid than that of the untreated callus cultures. Tetracosanoic acid was absent in the untreated calli. However, the leaf and EMS-treated callus cultures synthesized this long-chain fatty acid. The presence of long-chain fatty acids such as hexacosanoic ($RT = 28.506$) and octacosanoic acid ($RT = 33.989$) was also detected in leaves by mass spectrometry (Table 1).

The synthesis of different fatty acids in the EMS-treated callus cultures was severely affected by an increase in the concentration of EMS. However, the content of a few fatty acids was higher in EMS-treated calli compared to that in

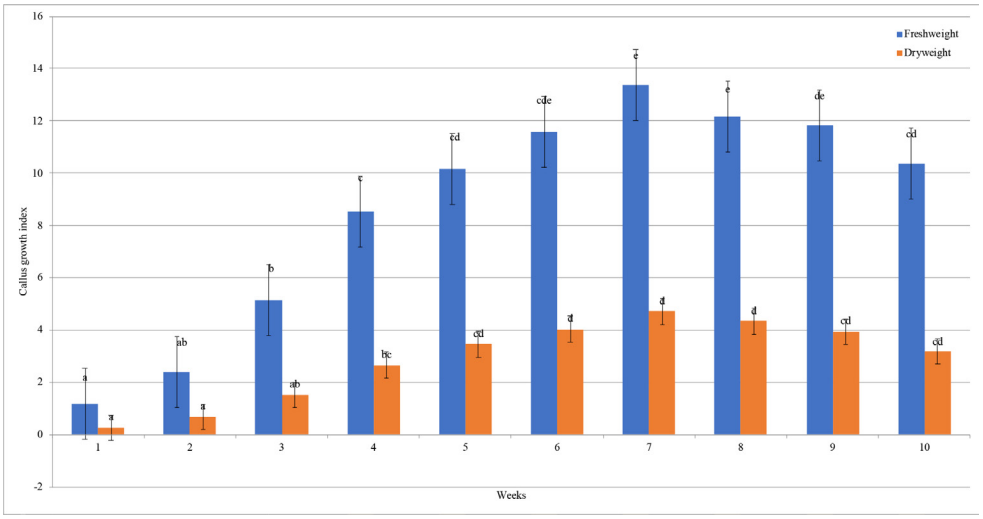

Fig. 4. Callus growth index obtained on MS medium supplemented with 2 µM BA + 2.5 µM 2,4-D from leaf explants of *P. oleracea*. Means followed by the same letters are not statistically significantly different according to Tukey's HSD test ($n = 12$; $p \leq 0.05$).

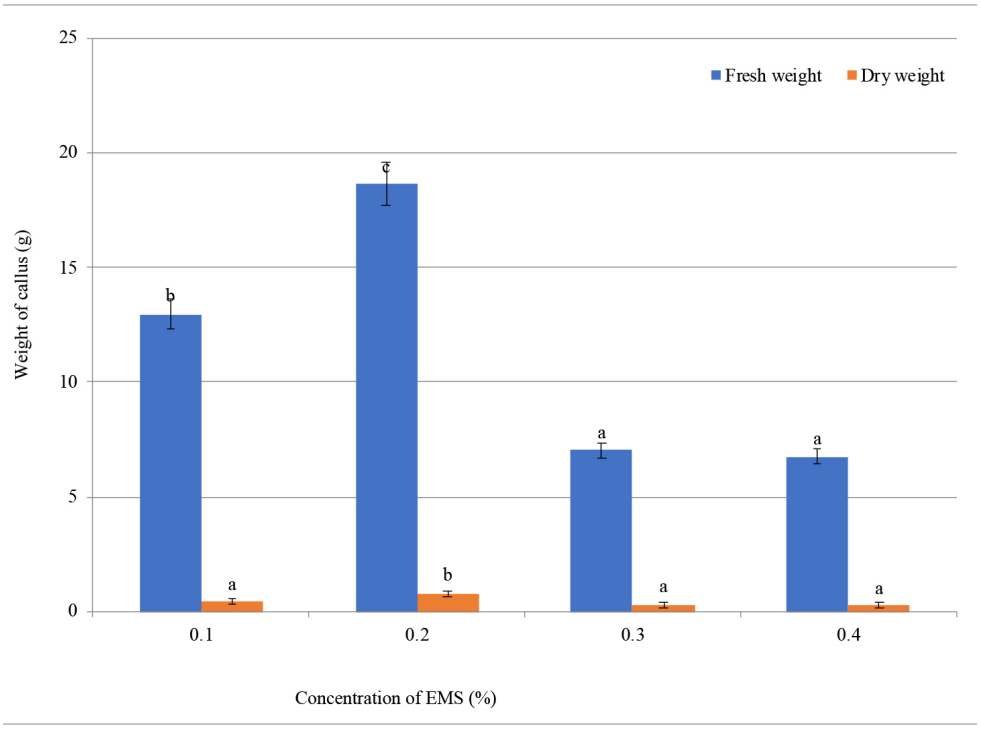

Fig. 5. Effect of EMS on induction and growth of callus on leaf explants of *P. oleracea* after 12 weeks. Means followed by the same letters are statistically not significant with Tukey's HSD test ($n = 12$; $p \leq 0.05$).

untreated calli. For the callus cultures obtained at 0.1% EMS, eicosanoic acid was 1.5 times and tricosanoic acid was 1.6 times higher than that in the untreated cultures. Linoleic acid was two times higher in the cultures of 0.1% EMS than in the untreated callus. The cultures with 0.2% EMS synthesized stearic acid, which was absent in untreated cultures, and the content of other PUFA's was reduced. Fatty acid synthesis was severely affected at a high concentration of 0.3% EMS, as different fatty acids were either absent or synthesized in low content (Table 1). These results indicated that P. *oleracea* leaves were rich in OFA, but its callus cultures synthesized fewer OFAs and the synthesis of long-chain fatty acids was triggered. EMS-treated cultures at low concentrations can synthesize a high content of specific fatty acids and long-chain fatty acids, indicating that variability in the fatty acid content can be induced at low concentration of EMS.

Discussion

In vitro *regeneration and callus induction from the explant*

The role of PGRs in plant tissue culture technique is crucial because their interplay determines the type of response elicited from the explant. The seminal work of Skoogs

Table 1. Fatty acids content (relative mean area % ± SE of the fatty acid peak) in leaves, untreated and EMS-treated callus cultures of *P. oleracea*. Means followed by the same letters are statistically not significant with Tukey's HSD test ($n = 3$; $p \leq 0.05$).

Fatty acid	Leaves	Untreated callus	Callus treated with EMS (%)		
			0.10	0.20	0.30
Pentadecanoic	1.04 ± 0.74 a	0.48 ± 0.03 a	0	0	0
Palmatic	8.51 ± 1.60 b	14.94 ± 0.36 f	7.60 ± 0.34 c	9.26 ± 0.69 b	2.73 ± 0.47 a
Heptadecanoic	0	2.64 ± 0.07 b	0	0	0
Oleic	0	4.43 ± 0.17 c	0	0	0
Stearic	2.98 ± 0.34 a	0	0	10.65 ± 1.22 b	0
Linoleic	3.25 ± 0.14 ab	5.77 ± 0.29 d	12.36 ± 1.16 d	0	0
Linolenic	17.72 ± 1.62 c	3.08 ± 0.16 b	0	0	0
Eicosanoic	1.03 ± 0.25 a	2.87 ± 0.38 b	4.55 ± 0.81 b	3.78 ± 0.23 a	0
Docosanoic	2.22 ± 1.10 a	7.07 ± 0.33 e	0.81 ± 0.02 a	1.15 ± 0.11 a	2.10 ± 0.13 a
Tricosanoic	0	3.08 ± 0.10 b	5.12 ± 0.37 bc	0	0
Tetracosanoic	0.71 ± 0.09 a	0	0.94 ± 0.14 a	0.83 ± 0.04 a	2.42 ± 0.35 a

and Miller (1957) demonstrated that the effects of auxin and cytokinin are quantitative (Melnyk 2023). A high concentration of auxin to low concentration of cytokinin was found to promote root formation, whereas a low concentration of auxin and high concentration of cytokinin favoured shoot bud or shoot formation in the explant. Similarly, a high concentration of both auxin and cytokinin favoured callus growth (Skoog, Miller 1957). Thus, varying the concentration of these PGRs can determine the regenerative fate of explants.

A similar quantitative relationship was observed in the present study, where a low concentration of auxin 2,4-D (0.5 µM) coupled with BA/KIN (10 µM) caused an organogenetic pathway such that shoots differentiated from the explants. The *in vitro* response to different species of *Portulaca* varies with the type and concentration of the PGRs used. In *Portulaca pilosa* optimal adventitious shoots were formed in medium supplemented with thidiazuron and 1-naphthalene acetic acid (Chen et al. 2020), whereas *in vitro* flowering was initiated in PGR-free medium (Xiong et al. 2021). The present study reports an induction of two or three shoots per explant in a medium supplemented with 10 µM BA and 0.5 µM 2,4-D, however a higher number of *in vitro* shoot formation in *P. oleracea* in a medium supplemented with BA and KIN was evident (Srivastava, Joshi 2021).

Accelerated callus induction was achieved with combined concentration of cytokinin (BA/KIN) and auxin (2,4-D). This prolific growth of calli is because both cytokinins and auxins are necessary for callus induction, as a coupled effect of both the PGRs induces cell division and cell growth (Irene et al. 2019). In addition, plants have an endogenous level of PGRs that are efficiently transported and cause enhanced callus growth (Sedaghati et al. 2018). The negligible callus formation at a single concentration of 2,4-D is similar to that in the studies with *P. pilosa* (Chen et al. 2020) . This indicates that the response of *Portulaca* to 2,4-D is uniform within the species, and that both cytokinin and auxin are necessary for callus induction.

Profuse non-embryogenic callus formation in BA + 2,4-D from leaf explants in the present study is similar to the results with *P. oleracea* (Sedaghati et al. 2018) and *Portulaca grandiflora* (Rossi-Hassani, Zyrd 1995; Rani et al. 2007). However, in other studies, callus induction was reported in *P. oleracea* with BA/KIN + indole-3-acetic acid/indole-3-butyric acid (Safdari, Kazimitabar 2010; Shekhawat et al. 2015). Similarly, callus induction in leaf explants of *P. grandiflora* was achieved by BA + 1-naphthalene acetic acid (Safdari, Kazimitabar 2009). Thus, *Portulaca* easily differentiates callus with BA and different auxins like 1-naphthalene acetic acid, indole-3-acetic acid, indole-3-butyric acid and 2,4-D, but the nature, texture and biomass of the callus may vary at the level of species or variety. The callus biomass induced in BA + 2,4-D was greater compared to that to KIN + 2,4-D. A study with *Allium ampeloprasum* also revealed that higher callus biomass can be obtained in BA + 2,4-D than in KIN + 2,4-D (Monemi et al. 2014).

In the combination of BA and 2,4-D, the maximum callus was achieved on medium supplemented with 2.5 µM BA and 2.5 µM 2,4-D (20.2 g per explant), and in the case of KIN and 2,4-D, optimal callus was obtained with 5 µM KIN and 5 µM 2,4-D (9.6 g per explant). This indicated that the growth of callus biomass was accelerated when the cytokinin to auxin ratio was balanced. Enhanced callus growth in a study with *Brassica oleracea* was obtained when cytokinin and auxin concentrations were identical (Ahmad, Spoor 1999). Similarly, optimum callus induction from petal explants (on a medium supplemented with 2 µM 2,4-D, 2 µM NAA, 2 µM KIN, 2 µM BA) and stem explants (on a medium supplemented with 2 µM 2,4-D and 2 µM KIN) was reported, with balanced cytokinin and auxin concentration ratios (Rossi-Hassani, Zryd 1995).

CGI is necessary to identify the time at which the maximum callus is produced in the cultures. The calli were harvested in the 7th week for fatty acid analysis. The CGI in both FW/DW indicated three stages; a lag phase, exponential phase and decline phase (Hafez et al. 2019). In the present study, the first stage during the 1st week was the lag phase, in which the CGI was low. Understandably, the slow growth occurred because the callus was transferred to fresh medium and adapted to a new *in vitro* environment. This stage was followed by an exponential phase, which accelerated from the 3rd week to the 7th week where the growth was rapid and CGI increased at a steady rate. The decline phase during the 8th to 10th week was characterized by a decrease in CGI and was accompanied by callus browning. The decline phase can be due to the depletion of salts in the medium, degradation of compounds synthesized in the callus, or the release of phenolics responsible for browning of cultures (Smith 2000; Irene et al. 2019).

Effect of EMS treatment on callus cultures

Mutations in plants can be induced by physical mutagens (such as gamma rays) or chemical mutagens. Among these chemicals, an alkylating agent such as EMS is a highly potent mutagen, as its mutagenic activity is due to the ethyl group that attaches itself to the guanine base. This alkylated guanine pairs with thymine, instead of cytosine, results in a G/C to A/T transition at the time of replication, or it can cause small deletions or rearrangements of the bases depending on the site of mutation (Watson et al. 2006). Thus, mutations in a plant cell can be induced by the application of mutagens at appropriate concentrations. The lethal dose 50 (LD_{50}) concentration varies among different plants, and it is important to determine this concentration for effective mutagen treatment of the plant. The LD_{50} concentration for *P. olereacea* has been determined to be 0.3% EMS (Srivastava, Joshi 2021). Therefore, in the present study, the callus was treated with 0.1 to 0.4% EMS (*v*/*v*) concentration. Profuse callus formation was evident

at a low concentration of 0.2% EMS, and callus biomass declined with an increase in the concentration of EMS. The survival percentage of explants and the average number of *in vitro* shoots induced from the explant were negatively affected with an increase in EMS concentration in *Vetiver* (Widoretno, Indriyani 2020). A similar effect of EMS, where the biomass was negatively affected by an increase in dosage of EMS, was reported (Kashtwari et al. 2018), indicating that it was due to sustained exposure of explant tissues to the mutagen, or the infiltration of the mutagen to the growing points causing toxicity to the explants.

Fatty acids in leaves and callus culture of P. oleracea

P. oleracea was considered a weed for a long time, but the presence of PUFA in the plant evoked the interest of nutritionists, and considerable progress has been made in the elucidation of its fatty acid fingerprints. Several studies have proved that different parts of *P. oleracea* plant are good sources of OFAs and other dietary fatty acids (Mousavi, Niazmand 2017; Desta et al. 2020; Farag et al. 2019). High linolenic acid content in *P. oleracea*. has been reported previously (Petropoulos et al. 2019). The present study also confirmed the presence of high content of linolenic acid in the leaves. The presence of long-chain fatty acids such as hexacosanoic (*RT* = 28.506) and octacosanoic acid (*RT* = 33.989) was also detected in the mass spectrometry analysis of the leaves.

Fatty acid composition in callus cultures of different plants has been studied (Rincón-Pérez et al. 2016; Rodryíguez-Hernández et al. 2018). An increase in the level of fatty acids in callus cultures of *P. oleracea* treated with jasmonic acid has been reported (Al-Bakr 2018). The present study determined the fatty acid profile of callus cultures and EMS-treated callus of *P. oleracea*. The synthesis of OFA (linoleic + linolenic acid) was more severely affected in callus cultures than in leaves. A negative corelation between fast growing *Arabidopsis* and *Acer* cell cultures and their linoleic and linolenic acid content has been shown (Mei et al. 2015). In particular, the enzymes FAD2 and FAD3 desaturases, required for the synthesis of linoleic and linolenic acid, respectively, had low activity in fast-growing cultures. This explains the low content of linoleic and linolenic acids in the calli in the present study.

EMS is frequently used to induce variability in plants, and has been useful for improving several agronomic traits in plants. Specifically, EMS has been used to influence the fatty acid composition of several plants (Hafeez et al. 2019; Hadebe et al. 2019; Subasi et al. 2023). The present study indicated that the synthesis of different fatty acids in the treated cultures was suppressed with an increase in EMS concentrations; however, the relative content of a few individual fatty acids was enhanced. Previous studies on fatty acid profiling of EMS-treated *in vitro* shoots on the same plant have also reported a similar result, where the synthesis of different fatty acids decreased with an increase in EMS concentration exposure to the explant (Srivastava, Joshi 2021). Thus, EMS directly affects biochemical pathways and alters the different fatty acids synthesized in the cultures. The synthesis of high stearic acid content (10.65%) in the cultures treated with 0.2% EMS is a desirable trait. Stearic acid is a saturated fatty acid, as studies have shown that, compared to palmitic acid, stearic acid in blood serum lowers the low-density lipoprotein and high-density lipoprotein cholesterol levels in humans (van Rooijen et al. 2021). Thus, an oil rich in stearic acid is healthier than that rich in palmitic acid.

The synthesis of fatty acids was severely affected by 0.3% EMS, which is understandable, as at this concentration the explant differentiated little callus. In addition, there was no statistically significant difference in the mean content of the fatty acids synthesised at this concentration (palmitic, docosanoic or tetracosanoic acids). The LD_{50} EMS dose in *P. oleracea* was 0.3% (Srivastava, Joshi 2021). The poor development of callus and low content of fatty acids synthesized at this dose indicated that there was 50% mortality of the cells, and significant synthesis of fatty acids was affected at this concentration. Therefore, in the treated concentrations, the overall profile of fatty acid synthesis was affected, but the individual content of some fatty acids (stearic, linoleic, eicosanoic and tricosanoic acids) was increased. Thus, there is a potential to increase the content of fatty acids at low concentration of EMS.

Conclusions

P. oleracea has the potential to induce high callus biomass, which can provide homogeneous biological material for the biotechnological extraction of dietary fatty acids. The extracted fatty acids can be used as food supplements and other minimally processed food products. The study provides a basic protocol of callus induction for further studies on cell suspension cultures, elicitations, precursor feeding and biotransformation; all of which are focussed on the enhanced level of fatty acid production. This study demonstrated that fatty acid content can be enhanced at low concentration of EMS.

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